60th Medical Group (AMC), Travis AFB, CA

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC)

FINAL REPORT SUMMARY

(Please type all information. Use additional pages if necessary.)

PROTOCOL #: FDG20110033A **DATE:** 1 May 2014

PROTOCOL TITLE: Role of Endothelial Differentiated Adipose-derived Stem Cells in Repairing Calvarial Critical Size Defects in the Laboratory Rat (*Rattus norvegicus*)

PRINCIPAL INVESTIGATOR (PI) / TRAINING COORDINATOR (TC): Maj Chris Gold

DEPARTMENT: General Surgery **PHONE #:** 423-5192

INITIAL APPROVAL DATE: 22 June 2011 **LAST TRIENNIAL REVISION DATE:** N/A

FUNDING SOURCE:

1. RECORD OF ANIMAL USAGE:

Animal Species:	Total # Approved	# Used this FY	Total # Used to Date
Rattus norvegicus	**100**	**0**	**71**

2. PROTOCOL TYPE / CHARACTERISTICS: (Check all applicable terms in **EACH** column)

___ Training: Live Animal
___ Training: non-Live Animal
_X_ Research: Survival (chronic)
___ Research: non-Survival (acute)
___ Other ( )

___ Medical Readiness
___ Health Promotion
___ Prevention
___ Utilization Mgt.
_X_ Other (Treatment)

___ Prolonged Restraint
___ Multiple Survival Surgery
___ Behavioral Study
___ Adjuvant Use
___ Biohazard

3. PROTOCOL PAIN CATEGORY (USDA): (Check applicable) ___ C _X_ D ___ E

4. PROTOCOL STATUS:

***Request Protocol Closure:**

___ Inactive, protocol never initiated

___ Inactive, protocol initiated but has not/will not be completed

_X__ Completed, all approved procedures/animal uses have been completed

5. FUNDING STATUS: Funding allocated: $ 249,912 Funds remaining: $ 72, 335

6. PROTOCOL PERSONNEL CHANGES:

Have there been any personnel/staffing changes (PI/CI/AI/TC/Instructor) since the last IACUC approval of protocol, or annual review? _X__ Yes ___ No

If yes, complete the following sections (Additions/Deletions). For additions, indicate whether or not the IACUC has approved this addition.

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE **16 JUL 2014**	2. REPORT TYPE **Final**	3. DATES COVERED **22 Jun 2011 - 16 Jul 2014**

4. TITLE AND SUBTITLE **FDG20110033A "Role of Endothelial Differentiated Adipose-derived Stem Cells in Repairing Calvarial Critical Size Defects in the Laboratory Rat (Rattus norvegicus).**	5a. CONTRACT NUMBER 5b. GRANT NUMBER 5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) **Maj Chris Gold, Dr. David Sahar**	5d. PROJECT NUMBER **FDG20110033A** 5e. TASK NUMBER 5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **Clinical Investigation Facility David Grant Medical Center 101 Bodin Circle Travis AFB, CA 94535**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) **Clinical Investigation Facility David Grant Medical Center 101 Bodin Circle Travis AFB, CA 94535**	10. SPONSOR/MONITOR'S ACRONYM(S) 11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Objectives: Calvarial bone defects are amongst the most common combat injuries. The treatment of large defects is difficult due to donor site limitations. Our aim in this study was to evaluate in vivo bone engineering in poly lactic-co-glycolic acid (PLGA) scaffolds seeded with endothelial and osteogenic differentiated adipose-derived stem cells (ASCs). Methods: Rat ASCs were induced into endothelial (ASC-endo) and osteogenic (ASC osteo) lineages. The optimal duration of endothelial cell differentiation was evaluated with flow cytometry analysis. Osteogenic differentiation was confirmed with alizarin red staining. Critical size (8 mm) defects were created in the calvaria of Lewis rats. The defects were treated with blank PLGA scaffolds (group I), PLGA scaffolds with undifferentiated ASCs (group II), PLGA scaffolds with ASC-osteo (group III), or PLGA scaffolds with ASC-endo (group IV). Bone healing in the defects with evaluated at 8 weeks postsurgery with micro-CT scans and histological staining with hematoxylin-eosin and Massons trichrome stains). Results: Micro-CT analyses of calvarial defects showed the highest bone mineral density in the ASC-osteo group, but there was no statistically significant difference between treatments and control ($p = 0.56$) (Fig 3). Photometric analysis of histology slides suggested a trend towards more bone formation in the ASC-osteo group, but there was no significant difference between treatments and control ($p = 0.13$) (Figs. 4 & 5). Conclusions: We were able to successfully differentiate ASCs into endothelial and osteogenic lineages and confirmed this using gene expression, protein expression, and histology. PLGA scaffold was a suitable medium for cell seeding. However, we were unsuccessful in producing significant new bone formation when osteocyte or endothelial cell differentiated ASCs were seeded separately on scaffolds in rat critical sized calvarial defects. These results suggest that ASC seeded PLGA scaffolds are unsuitable for repair of calvarial critical sized defects in rats.

15. SUBJECT TERMS **US Air Force, Medical Service, Medical Research, Graduate Medical Education**					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**UU**	**5**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

ADDITIONS: (Include Name, Protocol function - PI/CI/AI/TC/Instructor, IACUC approval - Yes/No)

Hakan Orbay, Post-doctoral scholar, IACUC approved.

DELETIONS: (Include Name, Protocol function - PI/CI/AI/TC/Instructor, Effective date of deletion)

_____None____

____

7. PROBLEMS / ADVERSE EVENTS: Identify any problems or adverse events that have affected study progress. Itemize adverse events that have led to unanticipated animal illness, distress, injury, or death; and indicate whether or not these events were reported to the IACUC.

- Dr. Brittany Busse was initially assigned to work on this project, but had to leave due to health related issues. Dr. Hakan Orbay was hired to resume the work, but some delays were encountered.
- Technical problems with the micro-CT scanner caused delays while the technician was not able to enter the US due to visa issues.
- Two animals died while the calvarial defect was being created under anesthesia at the beginning of the study. This resulted in the total animals in each group being changed from 9 to 7. This was reported to the IACUC.

8. REDUCTION, REFINEMENT, OR REPLACEMENT OF ANIMAL USE:

REPLACEMENT (ALTERNATIVES): Since the last IACUC approval, have alternatives to animal use become available that could be substituted in this protocol without adversely affecting study or training objectives?

None.

REFINEMENT: Since the last IACUC approval, have any study refinements been implemented to reduce the degree of pain or distress experienced by study animals, or have animals of lower phylogenetic status or sentience been identified as potential study/training models in this protocol?

A change in analgesia delivery was made from twice daily buprenorphine injections for two days to a single injection of a depot form of buprenorphine. This change will provide more consistent analgesia and will result in less distress for the animals as they will not need to be handled for repeat injections.

REDUCTION: Since the last IACUC approval, have any methods been identified to reduce the number of live animals used in this protocol?

None.

9. PUBLICATIONS / PRESENTATIONS: (List any scientific publications and/or presentations that have resulted from this protocol. Include pending/scheduled publications or presentations).

- 24th Annual Surgery Resident Research Symposium, 2014, UC Davis.
- Plastic Surgery Research Council Meeting, 2014, New York City.
- 25th Annual Surgery Resident Research Symposium, 2015, UC Davis.
- California Society of Plastic Surgeons Annual Meeting, 2015.
- Military Health System Research Symposium, 2014.

10. Were the protocol objectives met, and how will the outcome or training benefit the DoD/USAF?

The objectives were met in that we successfully: 1) created critical sized calvarial defects that did not spontaneously heal, 2) differentiated ASCs into endothelial and osteogenic lineages, and 3) seeded undifferentiated and differentiated cells onto PLGA scaffolds. However, we were unsuccessful in producing significant new bone formation when osteocyte and endothelial cell seeded scaffolds were placed separately in rat critical sized calvarial defects. These results suggest that ASC seeded PLGA scaffolds are unsuitable for repair of calvarial critical sized defects in rats.

11. PROTOCOL OUTCOME SUMMARY: (Please provide, in "ABSTRACT" format, a summary of the protocol objectives, materials and methods, results - include tables/figures, and conclusions/applications.)

Objectives: Calvarial bone defects are amongst the most common combat injuries. The treatment of large defects is difficult due to donor site limitations. Our aim in this study was to evaluate in vivo bone engineering in poly lactic-co-glycolic acid (PLGA) scaffolds seeded with endothelial and osteogenic differentiated adipose-derived stem cells (ASCs).

Methods: Rat ASCs were induced into endothelial (ASC-endo) and osteogenic (ASC osteo) lineages. The optimal duration of endothelial cell differentiation was evaluated with flow cytometry analysis. Endothelial differentiation was confirmed with Factor-VIII, vonWillobrand Factor (vWF), and CD-31 immunofluorescence staining and qRT-PCR for Factor-VIII, nitric oxide synthetase, vWF, CD-31, VEGFR-1, versican, ICAM-2, desmogein-1,and integrin α4 genes. Osteogenic differentiation was confirmed with alizarin red staining. Critical size (8 mm) defects were created in the calvaria of Lewis rats. The defects were treated with blank PLGA scaffolds (group I), PLGA scaffolds with undifferentiated ASCs (group II), PLGA scaffolds with ASC-osteo (group III), or PLGA scaffolds with ASC-endo (group IV). Bone healing in the defects with evaluated at 8 weeks postsurgery with micro-CT scans and histological staining with hematoxylin-eosin and Masson's trichrome stains).

Results: The expression levels for Factor-VIII, nitric oxide synthetase, vWF, CD-31, VEGFR-1, versican, ICAM-2, desmogein-1,and integrin α4 genes were increased during the second week of ASC differentiation in vitro (Fig. 1A). ASC-endo cells were positive for Factor-VIII, vWF, and CD-31 antibodies in vitro (Fig 1B). ASC-osteo cells stained positive for alizarin red in vitro (Fig 2). Micro-CT analyses of calvarial defects showed the highest bone mineral density in the ASC-osteo group, but there was no statistically significant difference between treatments and control ($p = 0.56$) (Fig 3). Photometric analysis of histology slides suggested a trend towards more bone formation in the ASC-osteo group, but there was no significant difference between treatments and control ($p = 0.13$) (Figs. 4 & 5).

Conclusions: We were able to successfully differentiate ASCs into endothelial and osteogenic lineages and confirmed this using gene expression, protein expression, and histology. PLGA scaffold was a suitable medium for cell seeding. However, we were unsuccessful in producing significant new bone formation when osteocyte or endothelial cell differentiated ASCs were seeded separately on scaffolds in rat critical sized calvarial defects. These results suggest that ASC seeded PLGA scaffolds are unsuitable for repair of calvarial critical sized defects in rats.

(PI / TC Signature)

28 July 2014
(Date)

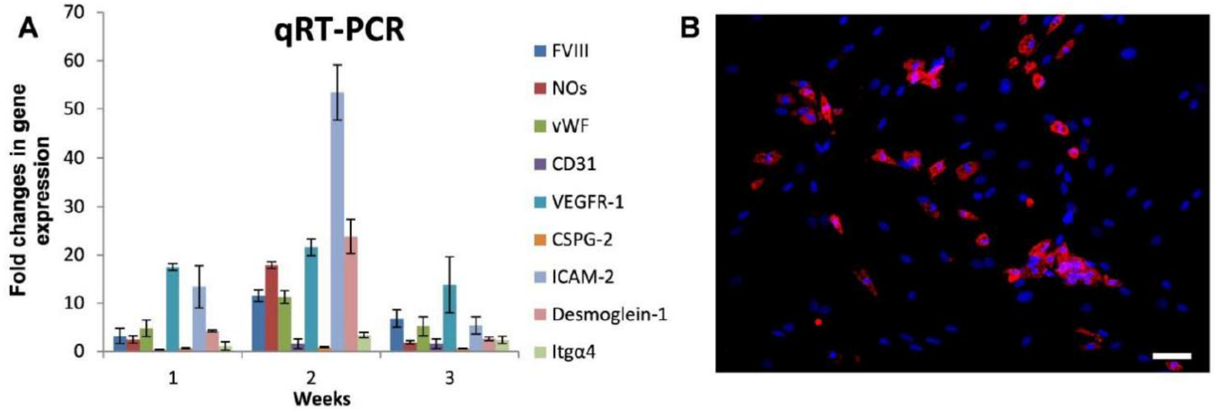

Figure 1. A. The changes in the gene expression levels of endothelial cell markers at weeks 1 ,2 and 3 during the differentiation of ASCs into endothelial cells. Bar graphs depicting means, the error bars are SDs. B. CD31 IF staining after 2 weeks of endothelial differentiation. AF546 was used as secondary Ab and nuclei were labeled with DAPI. Microbar 100μm.

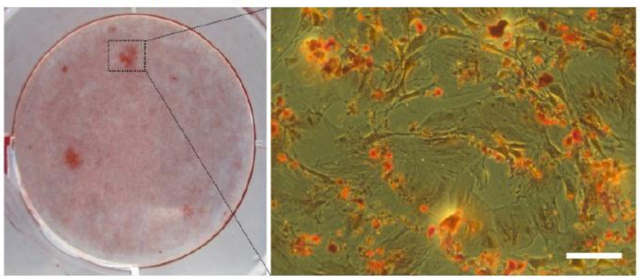

Figure 2. Alizarin red staining was performed to confirm the osteogenic differentiation of ASCs. Microbar 100μm.

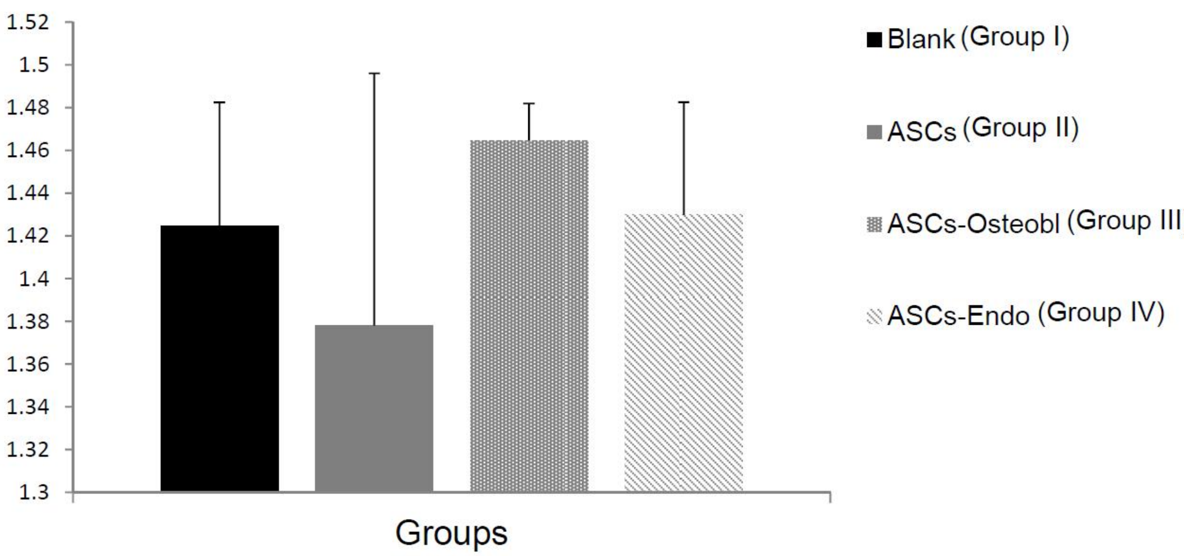

Figure 3. Micro-CT analysis of bone healing. There was no bone healing in the control animals-no treatment. Bar graphs depicting means, the error bars are SDs. P=0.56

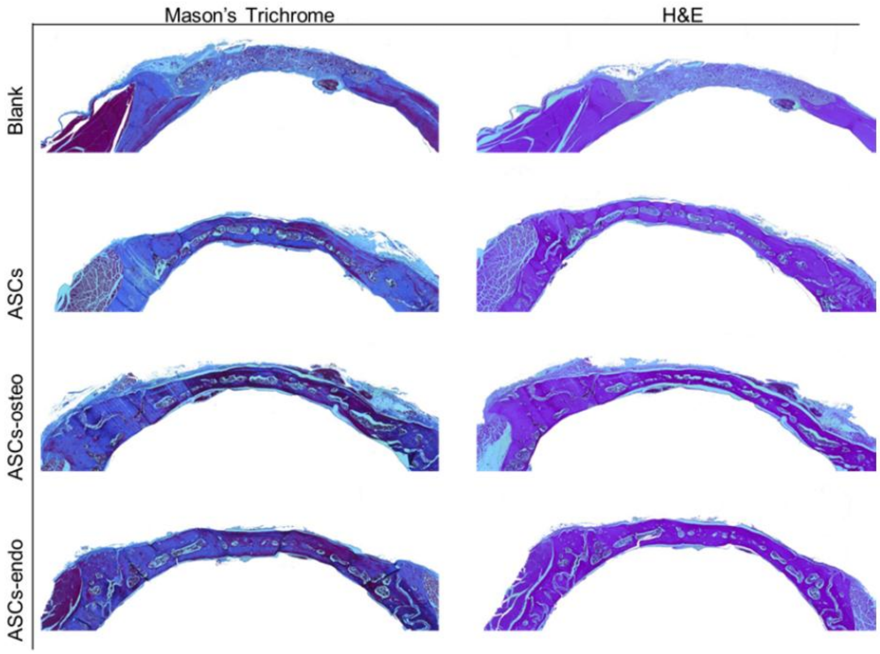

Figure 4. Eight weeks after the operation the animals were euthanized for histological evaluation with Mason's trichrome and HE stains. The calvarial defect was bridged with mainly fibrotic tissue in blank scaffold group whereas there was bicortical bone structure in varying amounts in the other groups.

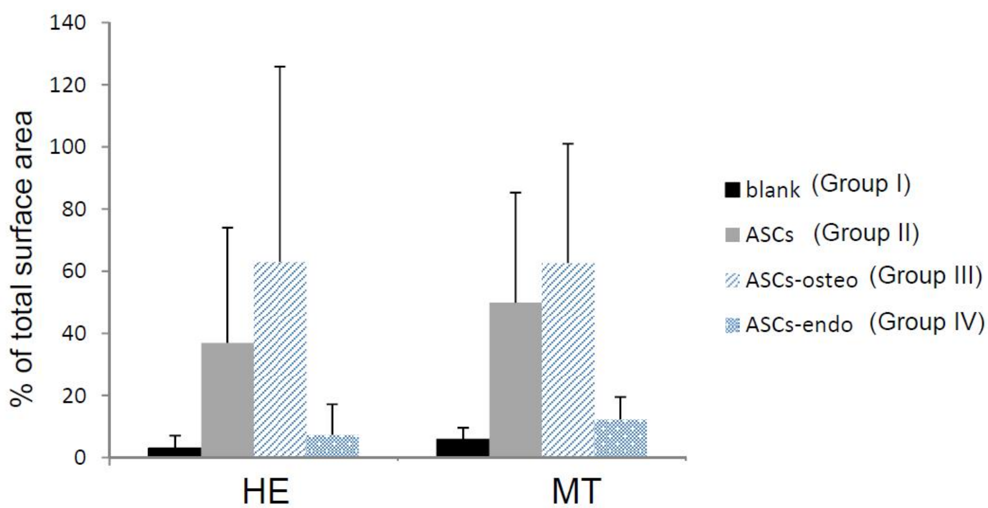

Figure 5. The graph shows the formation of the new bone in HE and MT stained slides from Group I (blank), Group II (ASCs), Group III (ASCs-osteo) and Group IV (ASCs-endo). The new bone formation is expressed as the percentage of the total surface area.